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The measurement of free iron in hemoglobin solutions as well as the measurement of total hemoglobin-bound iron are important validation assays for large-scale production of hemoglobin-based oxygen carriers. In addition, the detection of iron released from hemoglobin by oxidation or reduction reactions provides information about the stability of iron in heme. These stability measurements may be important in the characterization of structurally modified hemoglobins and can be conducted using spectrophotometric assays based on ferrozine, a chelator of ferrous iron. Techniques for the measurement of free iron in hemoglobin solutions, total hemoglobin-bound iron, and iron released from hemoglobin are described in detail.

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SECTION X

RELEASE OF IRON FROM HEMOGLOBIN

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In normal physiological states, iron is found almost exclusively bound to protein molecules in specific subcellular compartments, and the concentration of free or labile iron is very tightly controlled.[1] When decompartmentalized, however, iron can contribute to the development of pathophysiological states through various mechanisms.[1,2]

One pathophysiological state that can result from increased concentrations of free iron is bacterial infection. Iron is an element that is essential for bacterial growth, and the normal homeostatic mechanism of combating bacterial infection is to create a state of hypoferremia.[3] In the presence of increased concentrations of free iron, the probability of rapid bacterial growth greatly increases, possibly resulting in sepsis.[3] A second state of pathophysiology that can arise from high concentrations of free iron is related to iron's ability to catalyze free radical reactions that can cause considerable damage to cellular constituents.[2,4] Iron is a transition element that normally participates in oxidative and reductive reactions, but its reactivity is directed and controlled by protein molecules.[5] Iron's redox activity, however, can continue in solution in a non-specific or site-specific but uncontrolled fashion, potentially damaging proteins, nucleic acids, and lipids.[6,7] Such cellular damage is mediated by highly reactive oxygen free

radicals, possibly the hydroxyl radical, generated through the iron-catalyzed Haber-Weiss reaction, which is represented by the following three reactions.[8]

$$O_2^{\cdot-} + Fe(III) \rightleftharpoons O_2 + Fe(II) \qquad (1)$$

$$2O_2^{\cdot-} + 2H^+ \longrightarrow O_2 + H_2O_2 \qquad (2)$$

$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + OH^- + \cdot OH \qquad (3)$$

Hemoglobin comprises the largest reservoir of iron in the human body (70-75%).[9] Ferrous iron, which has six coordination positions, is bound in the heme pocket of hemoglobin by the four pyrrole nitrogen atoms of the protoporphyrin moiety, forming a tetradentate chelate with iron.[10] The imidazole residue of the proximal histidine of the heme pocket occupies the fifth coordination position. The sixth coordination position of iron is open in non-hemichromic hemoglobin and is free to bind ligands distal to the heme moiety.[10]

To function as a redox couple, iron must have at least one free coordination position,[11] which is the case for hemoglobin-bound iron. Consequently, it was hypothesized that hemoglobin-bound iron can catalyze the Haber-Weiss reaction while still bound in the heme pocket.[12,13] However, other studies have suggested that iron cannot act as a biological Fenton reagent in the heme pocket and that the redox reactive species is either free iron or heme outside the heme pocket.[14,15,16] Despite the fact that iron is bound tightly to hemoglobin, it can be liberated under specific circumstances, yielding

a source of "free, reactive iron".[2] The term "free, reactive iron" is meant to imply only that iron has been released from heme and ligated to another moiety, perhaps the distal histidine in the heme pocket. Once outside the heme pocket, iron can be translocated to other iron-binding sites, i.e., inorganic chelators, lipids, other proteins, or nucleic acids, where it can catalyze the Haber-Weiss reaction (see Eqs. 1-3).[1,2] Situations in which the heme moiety itself is released from hemoglobin, instead of iron, are under study by other investigators.[17]

Thus, the measurement of free iron in a variety of solutions will permit prediction and management of potential biological hazards from iron-catalyzed free radical reactions. In addition, the ability to measure iron released by hemoglobin in different experimental paradigms facilitates studies of the effects of structural modifications of the hemoglobin molecule on the stability of iron in heme. And finally, the measurement of free iron concentrations in solutions of hemoglobin is an important part of quality control/quality assurance of large-scale production of hemoglobin-based oxygen carriers and is included as one of the physicochemical variables in the Points to Consider for hemoglobin-based oxygen carriers.[18]

preparation of hemoglobin solutions

Two different preparations of hemoglobin were used to characterize the assays described in this chapter. The first, designated "stroma free hemoglobin" (SFHb), is derived from outdated human erythrocytes that are subjected to gentle, hypo-osmotic

lysis, followed by ultrafiltration, which removes all residual membranes ("stroma"). This preparation contains hemoglobin as well as other components of the red blood cell. The second type of hemoglobin, designated adult human hemoglobin (HbA_0), is produced by subjecting stroma free hemoglobin to high pressure liquid chromatography, yielding a chromatographically pure hemoglobin solution. Both types of hemoglobin were prepared according to techniques previously described.[19,20,21] Cyanomethemoglobin was prepared according to the method of Di Iorio.[22] All hemoglobin samples were supplied by the Letterman Army Institute of Research hemoglobin production facility.

detection of free iron in solutions of purified hemoglobin

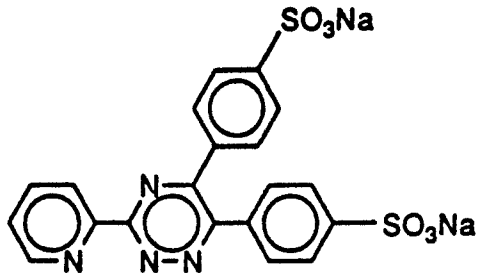
Outside the red cell membrane, when hemoglobin is processed or purified, small amounts of free iron can be detected as Fe(II) using the following method. All hemoglobin samples are assayed in duplicate; 250 µl of each hemoglobin solution is added to a 12 x 75-mm disposable borosilicate glass centrifuge tube, to which 250 µl of 20% trichloroacetic acid (TCA) (w/v) is added. The tubes are then centrifuged in a clinical centrifuge (Sero-fuge II, Becton Dickinson, Rutherford, NJ) to pellet the precipitated protein. From the supernatant, 250 µl are transferred to a new 12 x 75-mm test tube containing 250 µl distilled water. Subsequently, 2.5 ml of Sigma Iron Buffer Reagent (Sigma Chemical Co., St. Louis, MO, Cat. # 565-1; hydroxylamine hydrochloride, 1.5% (w/v) in acetate buffer, pH 4.5, with added surfactant)[23] is added to each tube, which is then mixed thoroughly. Finally, 50 µl of Sigma Iron Color

Reagent (Cat. # 565-3; ferrozine, 0.85% (w/v) in hydroxylamine hydrochloride)[23] is added to each tube, and the color is developed for 30 min at 37°C or 60 min at room temperature. The content of each tube is transferred to a disposable 4.0-ml cuvette and read at 560 nm. After correcting for dilution, data are expressed as µg iron/g hemoglobin.

A standard curve is constructed using the Sigma Iron Standard (Cat. # 565-5; 500 g/dl in hydroxylamine hydrochloride),[23] which is a 500 µg/dl solution of iron in hydroxylamine hydrochloride solution. Six standards, ranging from 0.25 to 2.5 µg, are used routinely and have been found to be reproducible over time (see Figure 1). The molar extinction coefficient calculated from this standard curve agrees with previously published estimates (approximately 28,000 l mole^{-1} cm^{-1}).[24,25,26] A number of different hemoglobin preparations have been tested including stroma-free red cell hemolysate, purified hemoglobin A_0, and hemoglobin covalently cross-linked between the alpha subunits.[27] Typical results are shown in Table 1. The values for iron content have been corrected for dilution.

Comments:

Since its initial characterization,[24] ferrozine has been used extensively as a chromophore to measure iron in a variety of assays.[25,26,28,29]



Scheme 1: Ferrozine

Since ferrozine binds only Fe(II), reducing reagents are required and several different compounds have been utilized, including ascorbic acid, thioglycolic acid, and hydroxylamine. Copper can interfere with ferrozine-dependent iron measurements, and copper-complexing agents have been included routinely in a number of iron assays.[25,26,28,29] Copper has not been found to be a significant contaminant of hemoglobin solutions, and copper-complexing agents have not been included in the present studies. Ferrozine also forms a colored complex with cobalt, which is also not a significant contaminant in our hemoglobin solutions; colored complexes are not formed between ferrozine and calcium or magnesium.[24] The original ferrozine assay for serum iron[25] has proven to be reliable but perhaps less sensitive than the bleomycin assay for free iron; however, numerous substances interfere with the latter assay, thus reducing its utility.[14] There is another assay for free iron based on the iron-binding chromophore Ferene S,[26] but since chromophore solutions have to be made fresh daily, the assay is cumbersome, which contributes to variability in the determinations.

The measurement of "free" or adventitious iron in hemoglobin solutions has been a controversial subject, with some investigators suggesting that non-heme iron might be produced as an artifact of the assay. Using the techniques described in this chapter, between 3.0-5.0% of the total iron is detected as non-heme. Significantly lower concentrations of non-heme iron were reported in another study of cross-linked hemoglobin (0.5-1.0 parts per million in 10 g% hemoglobin solutions).[30] A major difference between the two assay systems is that the assay described here utilizes an acid precipitation step, but the latter study separates hemoglobin from "free" iron by ultrafiltration in the presence of diethylenetriaminepentaacetic acid. An acid labile pool of iron associated with hemoglobin ("human blood") has been reported, and despite the fact the this earlier study used considerably more harsh conditions of acid treatment (16 hr at 37°C in 0.8% HCl), the amount of non-heme iron recovered (3.4-7.0% of total iron) was in the same range as those reported in this chapter.[31] One additional study using an acid precipitation step reported 3.4% non-heme iron in fresh bovine blood.[32] Consequently, the acid precipitation step used in the assays described in this chapter may facilitate the detection of non-heme iron present in hemoglobin solutions.

To determine whether the iron assay itself influences the recovery of non-heme iron, the amount of free iron in a hemoglobin solution was determined and defined as the background level. Subsequently, micromolar amounts of reduced and oxidized iron were added to the hemoglobin solution and total recovery was assessed. Fe(II) (as $FeSO_4$) was added to hemoglobin, which was then subjected to the acid denaturation

and the iron assay procedures described under "detection of free iron in solutions of purified hemoglobin"; all added iron was recovered and detected above the background level. However, if iron was added as Fe(III) (as $FeCl_3$), only the background level of iron was detected; none of the added Fe(III) was recovered. These results suggest that if "non-heme" iron in hemoglobin solutions is present in the oxidized form, it will not be detected by the assay system used for the current experiments. In addition, these data indicate that the labile iron pool in hemoglobin solutions may be present primarily as the reduced species of iron.

The results of the previous series of experiments led us to try to pre-form the ferrozine-iron complex by adding ferrozine to the hemoglobin solution prior to acid precipitation, predicting that the ferrozine-Fe(II) complex would survive precipitation and could be quantified in the protein-free supernatant. However, no complex was recovered. Hemoglobin A_0 also was added to a pre-formed complex of ferrozine and Fe(II), after which the hemoglobin was precipitated by TCA or removed by ultrafiltration. None of the ferrozine Fe(II) complex could be detected in solution after the hemoglobin was removed. These results suggest that either the ferrozine-Fe(II) complex binds to hemoglobin, or it is physically enmeshed in the denatured protein, which carries it into the pellet upon centrifugation.

Other experiments were conducted to determine whether iron complexing agents might influence the recovery or detection of non-heme iron in hemoglobin solutions. Ethylenediaminetetraacetic acid (EDTA), histidine, and citrate, were added at two

different concentrations, 0.1 and 1.0 mM, to the hemoglobin solutions prior to precipitation. The iron recovered when the assay was conducted in the presence of citrate and histidine increased above control levels at both concentrations. EDTA had no effect at the lower concentration, but iron recovery as detected by ferrozine was completely inhibited at the higher concentration. These results indicate that agents that interact with or bind iron may significantly influence the recovery of non-heme iron from hemoglobin solutions.

In summary, hemoglobin solutions seem to contain a pool of labile iron that can be quantified by using ferrozine. Apparently, the exact size of this pool can be influenced by reagents that interact directly with hemoglobin or iron. Consequently, constituents present in the final formulation of a hemoglobin solution may significantly influence the use of this assay system to measure non-heme iron.

spectrophotometric measurement of total hemoglobin-bound iron

In an attempt to release and measure all iron bound by hemoglobin, we modified the technique of Fish,[29] which entails an acid-permanganate-mediated digestion of the protein, which releases all protein-bound iron and converts it to Fe(III). The chromophore is then added in the presence of high concentrations of reducing agents, which convert Fe(III) back to Fe(II), which is necessary for the formation of an iron-chromophore complex. Five-hundred microliters of a solution of 0.6 N HCl in 2.25% (w/v, 0.142 M) $KMnO_4$ are added to a 50-µl sample of a 1.6 mM hemoglobin solution.

After digestion for 2 hours at 60°C, the sample is subjected to centrifugation (Micro-Fuge II, Becton Dickinson), the supernatant, which tests negative for iron, is removed, and the pellet is dried thoroughly. A 2.5-ml aliquot of Sigma Iron Buffer Reagent (Cat. # 565-1; hydroxylamine hydrochloride, 1.5% (w/v) in acetate buffer, pH 4.5, with added surfactant)[23] is added to each tube, which is then subjected to thorough mixing. This reagent accomplishes a complete solubilization of the pellet. Finally, 50 µl of Sigma Iron Color Reagent (Cat. # 565-3; ferrozine, 0.85% (w/v) in hydroxylamine hydrochloride)[23] is added to eacn tube, and the color is developed for 30 min at 37°C or 60 min at room temperature. The results of such a determination are shown in Figure 2. The calculated iron content, based upon the dilution of all hemoglobin solutions to a starting concentration of 1.6 mM (monomer), was 4.48 µg/50 µl, and the concentrations detected for HbA_0, SFHb, and cyanomethemoglobin were 4.35, 5.01, and 4.51 µg/50 µl, respectively.

Comments:

The total iron measured in hemoglobin solutions with this method corresponds well with theoretical calculations based on the actual concentration of hemoglobin. The spectrophotometric measurement of SFHb iron was higher than estimated by calculation; however, SFHb contains significant amounts of other iron-containing proteins, e. g., catalase and peroxidases, which may have inflated the final measurement. This technique has also been used to quantify the total iron content of

different organs. In summary, a rapid and accurate determination of the total iron content of a hemoglobin solution can be made using the chromophore ferrozine.

the effect of oxidants and reductants on the release of hemoglobin-bound iron

Significant quantities of iron, liberated from hemoglobin by both oxidative and reductive events, can be measured spectrophotometrically. The study of this phenomenon may provide important information about the reactivity of the iron or heme moieties of different hemoglobin molecules under conditions that may be present in disease states or trauma. These experiments may provide insight into the possible toxicity of cell-free hemoglobin solutions.

The loss of iron from oxyhemoglobin through oxidation reactions has been demonstrated previously[14,30,33,34] and can be measured easily on a routine basis. Incubations are conducted in 12 x 75-mm disposable borosilicate glass test tubes in a 1.0-ml final volume with the following constituents at their final concentration: phosphate buffer, pH 7.4, 10.0 mM; hemoglobin (monomer), 160.0 μM; and organic or inorganic peroxides or reducing agents or other effectors at varying concentrations. The reaction is initiated by the addition of the effector reagents, and incubations are conducted for 30-60 minutes at 37°C. At the end of the incubation, protein is precipitated by the addition of 250 μl of TCA (w/v). After centrifugation to pellet the precipitated protein, 500 μl of the supernatant are removed and transferred to another

12 x 75-mm test tube which contains 2.5 ml of Sigma Iron Buffer Reagent (Cat. # 565-1). After a thorough mixing, 50 µl of Sigma Iron Color Reagent (Cat. # 565-3) are added, followed by a second thorough mixing, and the color is allowed to develop for 30 min at 37°C or 60 min at room temperature. A standard curve ranging from 0.25 to 2.5 µg iron is included in each assay, and samples are analyzed spectrophotometrically at 560 nm.

The assay procedure can be modified to utilize microtiter plates and a microtiter plate-reader, which allows spectrophotometric measurements in 96 wells simultaneously. Hemoglobin incubations are conducted in quadruplicate in a 200-µl final volume in "U-bottom" 96 well plates (Falcon 3910, Becton Dickinson, Oxnard, CA). When possible, reagents are added using repeat pipettors, which greatly facilitates the preparation of each incubation mixture. In this modified assay, reactions are initiated by the addition of 20 µl of 1.6 mM hemoglobin (per monomer), and after the microtiter plate is covered with a sheet of Parafilm®, incubations are conducted at 37°C in a tissue culture incubator for one hour. At the end of the incubation, 25 µl of 20% TCA is added to each well to precipitate the protein, which is then pelleted by centrifugation at 2500 rpm in a microplate carrier for a tabletop centrifuge (Sorvall RT-6000B, Newton, CT). Following centrifugation, 50 µl of the supernatant from each well is transferred to a second, flat-bottomed microtiter plate (Falcon 3040), in which each well contains 200 µl of Sigma Iron Buffer Reagent (Cat. # 565-1). Finally, 10 µl of a 50% solution of Sigma Iron Color Reagent (Cat. # 565-3) is added to each well, and the iron-

chromophore complex is allowed to reach equilibrium during a one-hour incubation at room temperature.

Standard curves for the microtiter-plate iron assay are constructed by adding to a separate series of wells 50 µl of Sigma iron standards containing 50.0, 200.0, 500.0, and 1000.0 µg/dl.

The results from a series of experiments are shown in Figure 3. Using both purified human HbA_0 and SFHb, from 3% to over 50% of the total iron, based on heme concentration, was released during a one-hour incubation at 37°C. With the exception of hydrogen peroxide incubated with SFHb, all peroxides, at concentrations twenty-fold higher than that of hemoglobin-bound iron, released significant quantities of iron. The release of iron from hemoglobin by ascorbic acid is concentration-dependent.

Comments:

The limited ability of hydrogen peroxide to release iron from SFHb may be due to the presence of residual red cell catalase in this preparation. The organic peroxides are evidently poor substrates for catalase and are capable of releasing virtually identical amounts of iron from both HbA_0 and SFHb. Ascorbic acid liberated iron from both HbA_0 and SFHb in a dose-dependent fashion, but there was less total iron released from SFHb than from HbA_0. A number of other effectors, particularly reductants, were tested for their ability to release hemoglobin-bound iron, and cysteine, reduced glutathione, and superoxide were particularly effective.[34] Finally, the presence or

absence of buffer as well as the type of buffer in incubations of hemoglobin solutions with various effectors can have a pronounced influence on iron release. In the absence of buffering capacity or in the presence of physiological saline, iron release is attenuated, but iron release is increased in the presence of Tris or phosphate buffer. A possible explanation for these results may be that Tris and phosphate are capable of interacting directly with iron, removing it from a hemoglobin binding site and increasing its solubility.

Previous studies have demonstrated that hemoglobin can serve as a pro-oxidant, and the results of the iron assays discussed here raise the possibility that hemoglobin's pro-oxidant effect may be mediated, in some circumstances, by non-heme iron released from hemoglobin. While non-heme iron may mediate the pro-oxidant effects of hemoglobin, the sequence of biochemical reactions resulting in the liberation of hemoglobin-bound iron is not clear. It is not known whether heme dissociation from globin precedes the release of iron or whether iron can be liberated directly from globin-bound heme. In fact, the sequence of biochemical reactions may actually differ depending on the reaction conditions. For example, the inclusion of lipids in the incubation might provide a hydrophobic binding site for heme and support heme release; whereas, the aqueous conditions of the incubations in the assay described here may increase the liberation of free iron.

In conclusion, as the use of hemoglobin-based oxygen carriers is pondered, more information on the stability, reactivity, and destiny of hemoglobin-bound iron will be

required. The iron measurement assays described in this chapter provide a simple, yet reliable, means of accomplishing this goal.

The opinions and assertions contained herein are the private views of the author and are not to be construed as official, nor do they reflect the views of the Department of the Army or the Department of Defense (AR 360-5).

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Figure Legends:

Figure 1: Standard curve of iron detected spectrophotometrically as a complex with the chromophore ferrozine. The data represent mean values ± standard deviation for 8 independent determinations.

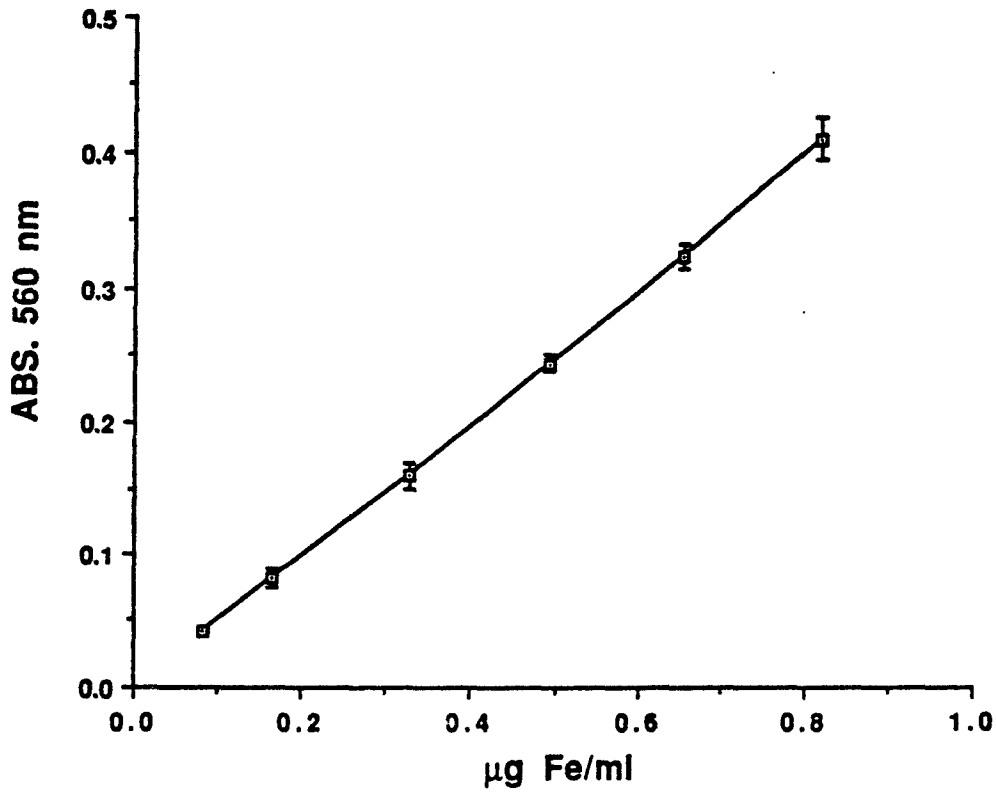
Figure 2: Measured iron concentration in different hemoglobin solutions compared with the theoretical iron content calculated from the concentration of hemoglobin (1.6 mM).

Figure 3: Iron released by incubation (60 min, 37°C) of 0.16 mM HbA_0 (A) or 0.16 mM SFHb (B) with 10.0 mM hydrogen peroxide (HP), 5.0 mM tertiary butyl hydroperoxide (TB), 5.0 mM cumene hydroperoxide (CU), or ascorbic acid at concentrations of 15.0 (AA1), 10.0 (AA2), 5.0 (AA3), or 2.0 mM (AA4). The condition labeled CON represents the amount of iron released by an incubation of hemoglobin and buffer without additional reagents. The data represent mean values ± standard deviation for 8 independent determinations.

TABLE 1: RESULTS FROM A REPRESENTATIVE ASSAY OF FREE OR LABILE IRON IN DIFFERENT HEMOGLOBIN SOLUTIONS

HEMOGLOBIN PREPARATION[a]	CONC. g/dl	Fe CONTENT µg Fe/g Hb
SFHb (91098)	8.38	115.6
HbA_0 (91040)	9.90	133.4
HbA_0 (91098)	6.83	136.8
ααXLHb (91119)	4.99	164.7

[a] Primary chromatographic component of adult human hemoglobin, HbA_0; stroma-free hemoglobin, SFHb; and hemoglobin cross-linked between the alpha subunits with diaspirin, ααXLHb. The numbers in parentheses represent the Julian date when that specific preparation was started at the Letterman Army Institute of Research production facility.



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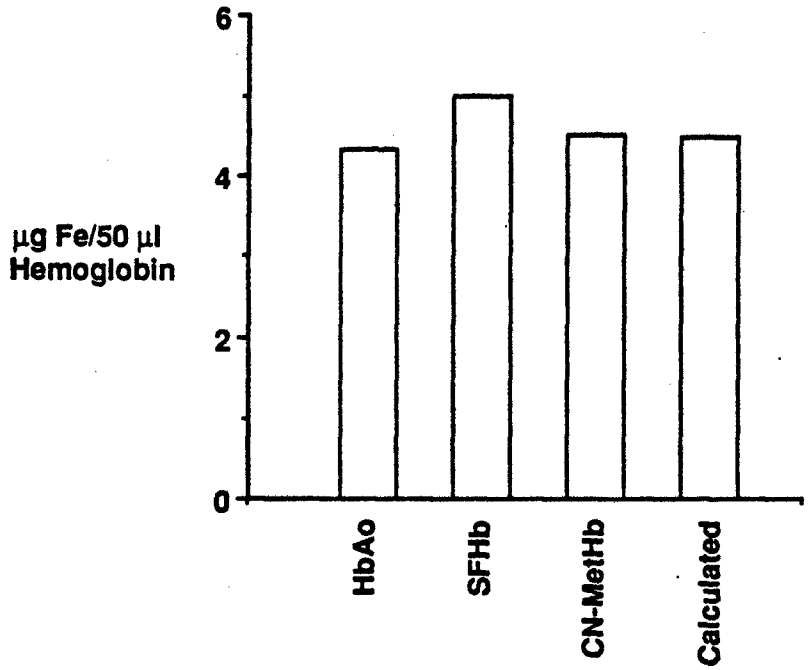

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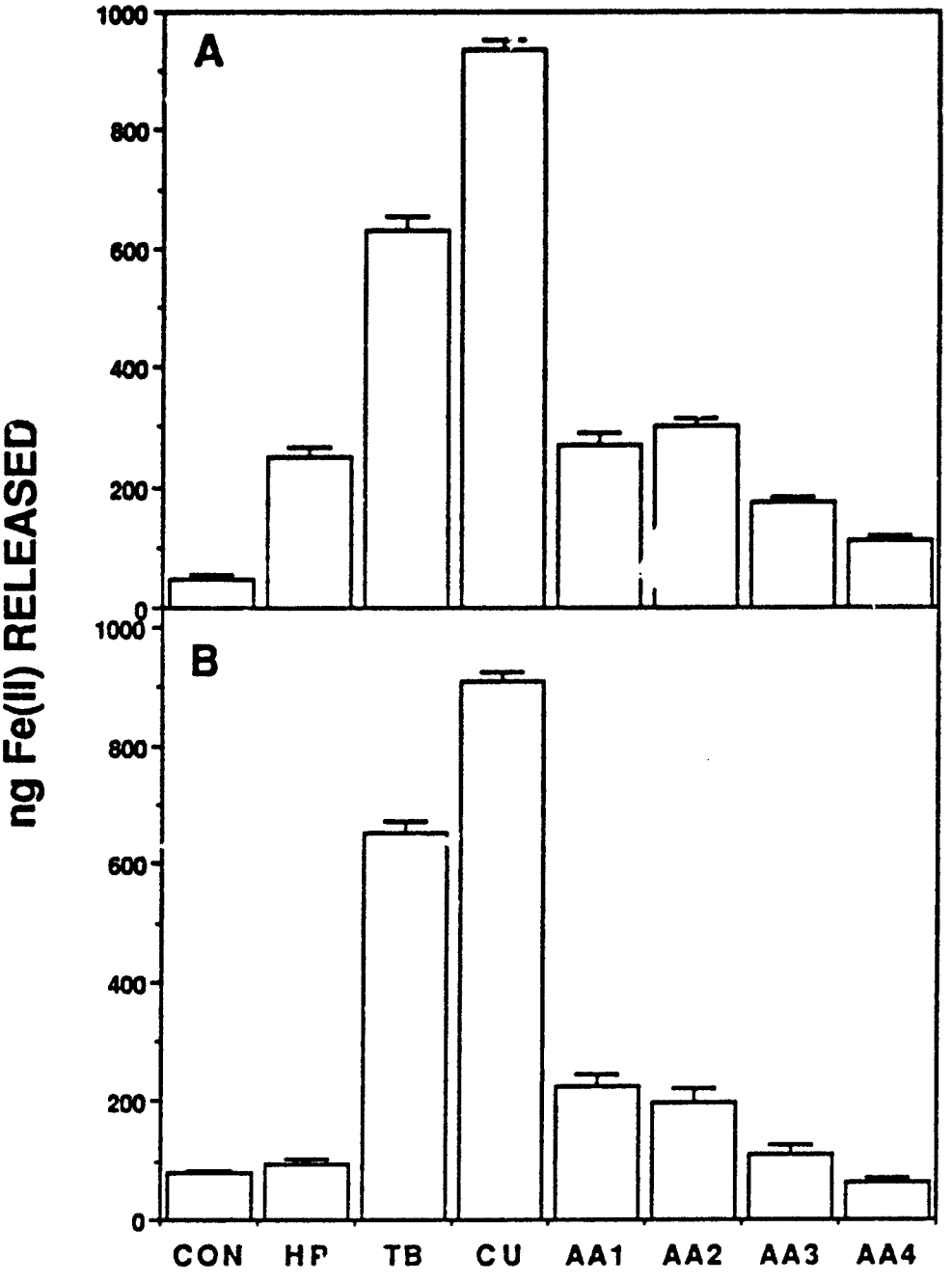
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